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14. ABSTRACT
Invariant natural killer (iNKT) cells are a unique population of immune cells that rapidly secrete a variety of cytokines upon activation and have been implicated in a autoimmune diseases, infection as well as cancer. In cancer, iNKT cells have generally been attributed potent anti-tumor functions but our studies using the murine 4T1 mammary carcinoma indicate a regulatory/ suppressive function that markedly affects response to treatment with radioimmunotherapy. We proposed to test the novel hypothesis that iNKT cells can be conditioned by the breast tumor environment to switch to an immunoregulatory phenotype. Specifically, three non-mutually exclusive hypothesis will be tested –1) that a lipid antigen derived from 4T1 tumor cells can be presented by dendritic cells and induce the preferential secretion of immunosuppressive cytokines; 2)that iNKT cells inhibit effector T cell priming by killing dendritic cells and 3) that immunoregulatory iNKT cells can promote the generation and maintenance of regulatory T-cells. Thus far, we have made significant progress in demonstrating that lipid antigen/s derived from 4T1 tumors can differentially modulate the maturational markers in dendritic cells. Mechanistic studies addressing effects on cross-priming are being planned for the second year.

15. SUBJECT TERMS
invariant natural killer T cells, breast cancer, immunology

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INTRODUCTION

Invariant natural killer T (iNKT) cells comprise a unique group of immune cells that specifically recognize lipid antigens presented in the context of CD1d molecules which leads to rapid and robust secretion of a wide range of Th1 and Th2 cytokines [1]. Although iNKT cells represent a small population of cells, their role in shaping the ensuing adaptive response puts them at a critical bridge between the innate and adaptive immunity. In cancer, iNKT cells are generally attributed a role in tumor immunosurveillance [2, 3] . However, using the 4T1 mouse model of metastatic breast carcinoma, we have seen that they assume a largely regulatory function especially in downregulating the therapeutic response to our regimen of combined local radiation and immunotherapy with CTLA-4 blockade [4]. It is likely that this regulatory function is imparted both by the host immune cells as well as tumor-derived factors. The goal of this project is to test the hypothesis that iNKT cells can be conditioned by the tumor microenvironment to switch to an immunoregulatory phenotype which inhibits the generation (priming) or effector function of anti-tumor T-cells. Three non-mutually exclusive hypotheses will be tested using the 4T1 model: 1) 4T1 tumor cell express a yet unidentified lipid antigen that, when presented directly or indirectly by dendritic cells to iNKT cells, can induce preferential secretion of immunosuppressive cytokines ; 2) iNKT cells inhibit effector T cell priming by killing dendritic cells that cross-present 4T1-derived tumor antigen and 3) immunoregulatory iNKT cell, by acting directly or indirectly through the secretion of cytokines, promote the generation and maintenance of regulatory T-cells.

BODY

For the first year, we proposed to execute experiments to determine whether direct or indirect presentation of a 4T1 –tumor derived ligand can induce a regulatory program in iNKT cells.

Task 1: Perform in vitro co-culture of 4T1 cells with iNKT cells

1a. Prepare 4T1 tumor lysates from tumor-bearing WT mice

1b. Enrich hepatic iNKT cells using CD1d tet (NIH) from WT mice

1c. Measure secreted cytokines by ELISA

We have previously detected CD1d mRNA in 4T1 cells by real-time RT-PCR [4] although surface expression by flow cytometry remains very low.

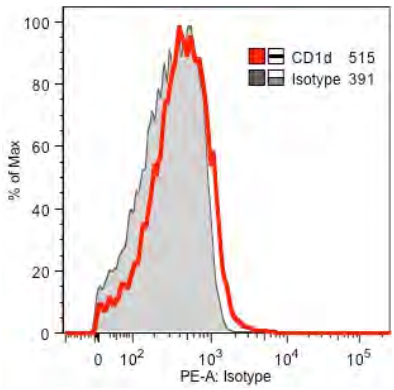

Figure 1: ***LOW LEVEL EXPRESSION OF CD1d IN 4T1 CELLS.*** *4T1 cells in culture were collected, washed and Fc-blocked prior to staining with PE-CD1d or PE-IgG2 (isotype control). The numbers representmean fluorescence intensity (MFI) of stained cells.*

However, low-level expression of CD1d has been shown to be sufficient for recognition by NKT cells [5, 6]. To determine if levels of CD1d expressed on 4T1 cells are sufficient for recognition by iNKT cells, we obtained an NKT hybridoma cell line (DN32.D3) from Masaki Terabe (Vaccine Branch at the NCI) with permission from Albert Bendelac (U Chicago) who originally developed the hybridoma [7]. DN32.D3 cells have been shown to recapitulate the cytokine response of native iNKT cells following activation by α-galactosylceramide [7] and have been used extensively by many investigators as a readout for NKT responses [8]. As shown in Figure 2, we observed a robust production of IL-2, IL-4 and IFN-γ when irradiated 4T1 (Ir-4T1) cells were loaded with α-galactosyl-ceramide (α-GalCer), indicating that low-level expression of CD1d in 4T1 cells was sufficient to induce the activation of DN32.D3 NKT cells. Furthermore, unloaded 4T1 tumor cells were unable to induce any significant activation of NKT cells, suggesting that direct tumor presentation of an endogenous lipid antigen may not be a key mechanism generating regulatory/suppressive NKT cells.

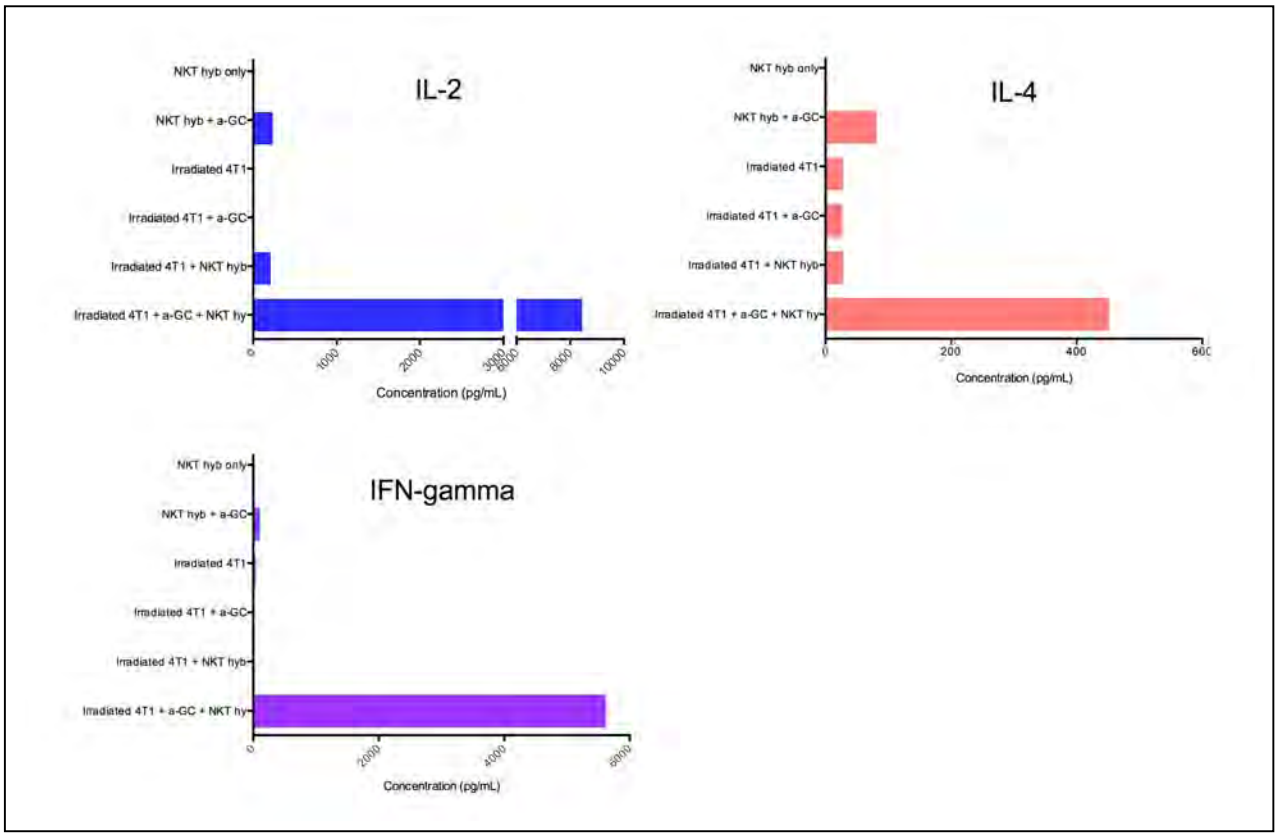

Figure 2: ***<u>4T1 CELLS CAN PRESENT α-GalCer AND ACTIVATE DN32.D3 NKT CELLS</u>****. Irradiated (12 Gy) 4T1 cells were loaded or not with α-GalCer overnight and washed prior to incubation with DN32.D3 NKT cells for an additional 72 hours. For each well, $2x10^5$ Ir-4T1 cells were cultured with 10^5 DN32.D3 cells. Secreted IL-2, IL-4 and IFNγ were measured by Cytometric Bead Assay (CBA, eBioscience).*

To verify that the cytokine response of DN32.D3 NKT cells is mediated by CD1d, we performed blocking experiments using a commercially available neutralizing antibody (clone 1B1, eBioscience). We used a single concentration of 100 μg at 5 different densities of irradiated, α-GalCer-loaded 4T1 cells. As a readout, secreted IL-2 was measured 72 hours after co-incubation with DN32.D3 NKT cells. We were unable to effectively block IL-2 production even with the lowest density of 10,000 irradiated 4T1 cells (Figure 3).

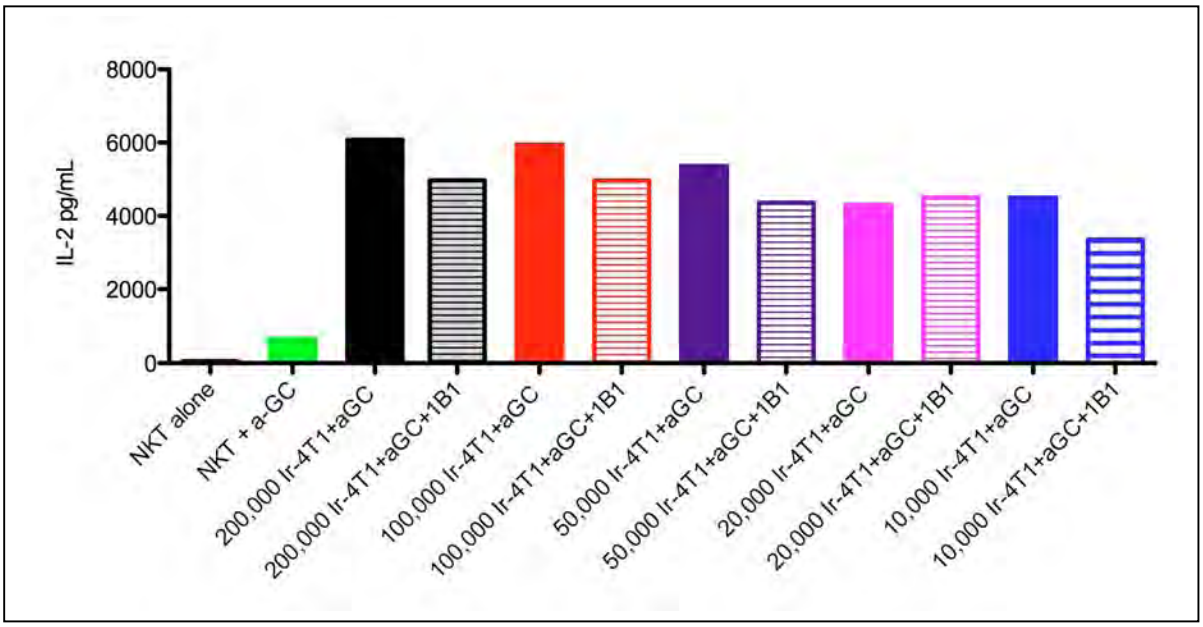

Figure 3: ***CD1d BLOCKING ANTIBODY (1B1) DOES NOT ABROGATE NKT RESPONSE TO α-GalCer-LOADED Ir-4T1 CELLS***. Increasing numbers of Ir-4T1 cells were loaded with α-GalCer (a-GC) overnight, washed and incubated with DN32.D3 NKT cells for 72 hours. In some wells, blocking antibody to CD1d (Clone 1B1) was added to a final concentration of 100 ug/mL.

We will need to confirm the blocking activity of the antibody using α-GalCer-loaded bone marrow-derived dendritic cells (BMDCs). Interestingly, we did not see a cell density-dependent decrease in secreted IL-2. These data are intriguing, and suggest the existence of an alternative receptor that can present lipid antigens to NKT cells.

Task 2: Assay indirect presentation by dendritic cells

2a. Loading of DC with 4T1 lysates or α-GalCer

2b. Measure secreted cytokines by ELISA

The regulatory programming of NKT cells in the tumor microenvironment could alternatively be induced by CD1d-expressing dendritic cells. Presumably, intratumoral dendritic cells would engulf tumor–associated lipid antigens that are subsequently cross-presented to NKT cells. A series of experiments were performed to study aspects of cross-presentation of tumor lipid antigens to NKT cells. First, we investigated whether

secreted factors from 4T1 cells can induce NKT cell activation. To this end, we supplemented BMDCs cultures with 4T1 and Ir-4T1 conditioned media prior to co-culture with DN32.D3 NKT cells. The results depicted in Figure 4 show a mild induction in NKT production of IL-2 and IFNγ when BMDCs were supplemented with 4T1 or Ir-4T1 conditioned media. Furthermore, an IL-2 but not IFNγ response was detected when Ir-4T1 cells themselves were co-cultured with BMDCs. However, these responses were miniscule when compared to the cytokine response to α-GalCer-loaded BMDCs. Interestingly, we found that NKT response to α-GalCer was comparable regardless of whether BMDCs were matured with LPS or not. This was surprising since BMDC maturation by LPS can enhance CD1d expression (Fig. 5) which would be expected to induce a more robust cytokine response from the hybridoma cells. Further investigation is needed to confirm the ability of 4T1-derived factors to affect iNKT cell activation by BMDCs, and its possible relevance in vivo, given the modest effect.

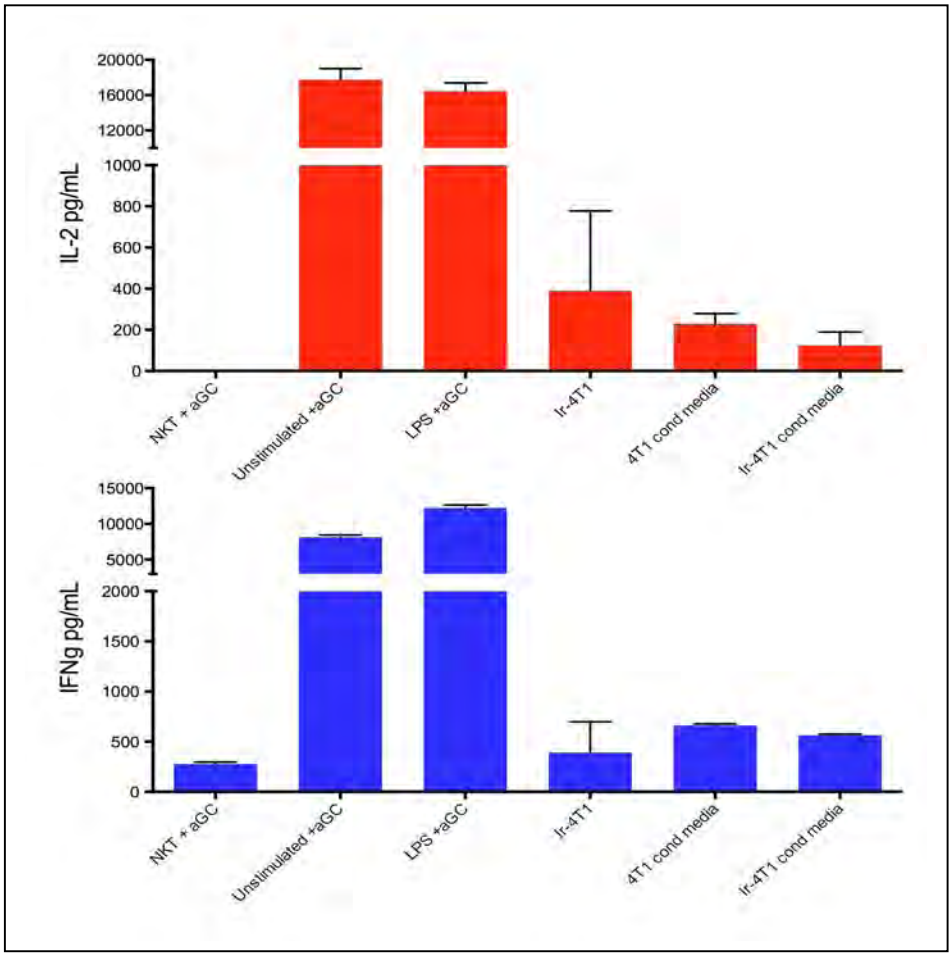

Figure 4. ***INDIRECT PRESENTATION OF 4T1-DERIVED SOLUBLE FACTOR CAN ACTIVATE NKT CELLS.*** Bone-marrow derived dendritic cells were obtained from bilateral femurs of wildtype BALB/c mice and cultured in GM-CSF using standard lab protocols. Using 24-well plates, $2x10^5$ BMDCs were supplemented with conditioned media from 4T1 or Ir-4T1 cells. In some wells, 10^5 Ir-4T1 cells were added. For controls, unstimulated and LPS-matured BMDCs were loaded with α-GalCer (aGC) overnight and washed prior to co-culture with hybridoma cells. NKT cells supplemented with α-GalCer (NKT+a-GC) were used as controls. After 72 hours, secreted IL-2 and IFNγ were measured by CBA as a readout of NKT cell activation. Values represent the mean± SEM of 3 wells.

Since DCs play a key role in orchestrating the activation of NKT cells, we assessed whether 4T1 tumor cells can alter the phenotypic and cytokine profile of DCs themselves. In an in vitro experiment, we measured DC activation marker CD86 as well as CD1d and representative cytokines secreted after 72 hours of incubation in several conditions. Compared to baseline unstimulated DCs, LPS-matured DCs expressed a fivefold increase in surface CD1d (Figure 5). The addition of conditioned media, either from 4T1 or Ir-4T1 did not affect CD1d expression. However, when Ir-4T1 cells were co-cultured with CD11c+ DCs, a twofold increase in CD1d expression was seen on DCs (*p<0.05)*. The same results were seen when DCs were loaded with Ir-4T1 lysates. BMDCs obtained from NKT-deficient mice showed comparable results.

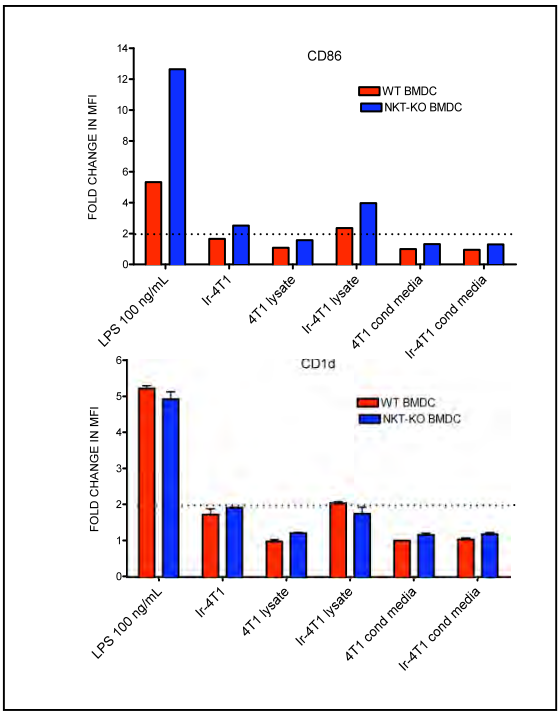

Figure 5: ***INCREASED CD1d AND CD86 EXPRESSION IN CD11c+ BMDCS LOADED WITH TUMOR CELL LYSATES.***
BMDCs were collected from WT BALB/c and NKT-deficient mice, cultured in GM-CSF using established protocols, and magnetically enriched for CD11c+ DCs using Miltenyi beads. DCs were loaded with lysates from 4T1 and Ir-4T1 or supplemented with conditioned media from 4T1 or Ir-4T1 cells. In some wells, 10^5 Ir-4T1 cells were added. After 72 hours, DCs were harvested and stained for surface CD86 and CD1d expression. Mean fluorescence intensities (MFI) were adjusted to baseline expression in unstimulated DCs. Values represent the mean of 3 independent wells. Dashed lines drawn at twofold increase in MFI were drawn as a point of reference. Supernatants were collected and stored at –80C for future cytokine quantitation by CBA

The same trends were seen for CD86 expression (Figure 5). A twofold increase in expression was observed only for DC cultures that were exposed to Ir-4T1 or loaded with lysates from Ir-4T1. Of note, the induction of CD86 by LPS and Ir-4T1 lysate in DCs from WT and NKT-deficient mice was different (*p=0.004)*, but these results should be confirmed in repeat experiments. Presently, the results of the analysis of cytokine release by DC are pending as of this writing. We plan to assay for IL-10, IL-12, IL-6 and TNF-α. Additionally, DC conditioned by incubation with 4T1 cells or their suprnatants/lysates will be co-cultured with DN32.D3 hybridoma cells to determine changes in the profile of cytokine secretion. We will be particularly interested the cytokine profile of DN32.D3 NKT cells when they encounter BMDCs loaded with Ir-4T1 tumor lysates.

We have also begun experiments outlined in Sp. Aim 2 that will investigate key differences in dendritic cell populations between WT BALB/c and NKT-deficient mice bearing subcutaneous 4T1 tumors. To determine quantitative differences in intratumoral dendritic cells, we inoculated both strains with 4T1 tumor cells on day 0. We collected tumors on days 13, 19 and 26 post tumor-inoculation for immunostaining of CD11c+ DCs on frozen tissue sections. The data showed a significant increase in the numbers of tumor-infiltrating CD11c+ DCs in NKT-deficient mice at all three timepoints examined.

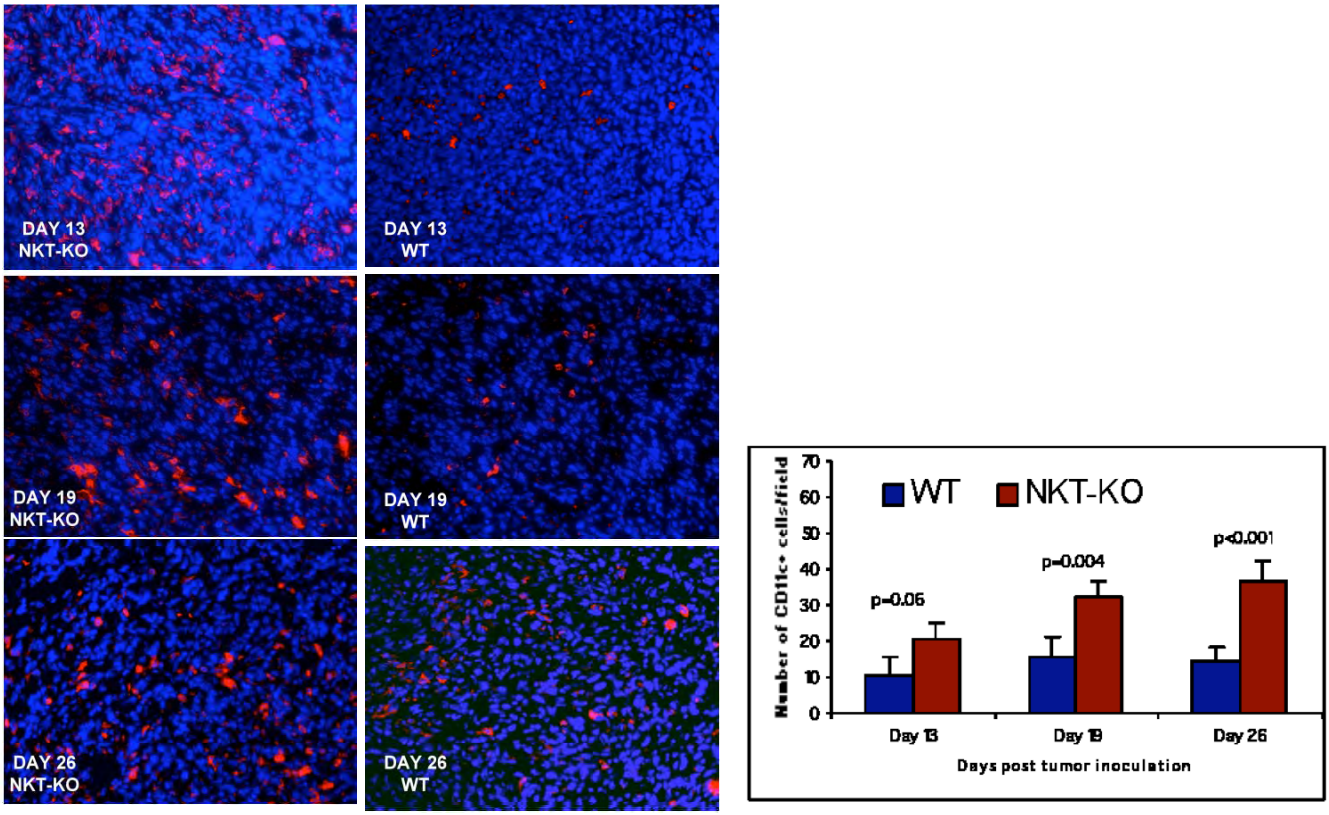

Figure 6: ***4T1 TUMOR-BEARING NKT-DEFICIENT MICE HAVE INCREASED NUMBERS OF TUMOR-INFILTRATING DCs****. WT BALB/c and NKT-deficient mice were inoculated subcutaneously with 4T1 tumor cells on day 0. Tumors were harvested on days 13, 19 and 26 and fixed in OCT media. Frozen tissue sections were stained for CD11c+ DCs. Counts were obtained from 3 fields per tumors. For each timepoint, 3 mice of each strain were used.*

We had also previously done a small preliminary experiment to determine phenotypic differences in tumor-infiltrating DCs between the BALB/c and NKT-deficient mice (Figure 7) which showed that the latter had DC exhibiting a more mature phenotype.

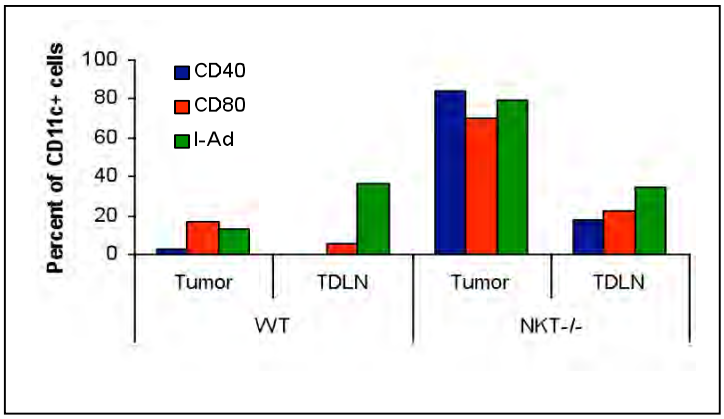

Figure 7. ***INTRATUMORAL DCs IN NKT-DEFICIENT MICE EXHIBIT A MORE MATURE PHENOTYPE****. BALB/c and NKT-deficient mice were inoculated with 4T1 tumors on day 0. Tumors and tumor-draining lymph nodes were harvested, enzymatically digested and magnetically enriched for CD11c+ DCs using Miltenyi beads. DCs were then stained for maturational markers CD40, CD80 and I-A^d by flow cytometry. Graphs show the percentage of positively stained CD11c+ DCs between the 2 strain of mice.*

Taken together, the increased numbers and maturation of DC within the tumor suggest that NKT cells could impair CD8 T-cell cross-priming to tumor antigen by targeting DC, which would be consistent with the increased CD8-mediated anti-tumor response seen in NKT-deficient mice given combined radioimmunotherapy [4]. As outlined in the statement of work for year two, studies will be done to systematically dissect how NKT cells may affect presentation and priming functions of dendritic cells within the context of 4T1 breast cancer.

KEY RESEARCH ACCOMPLISHMENTS

- Showed that 4T1 breast tumor cells loaded with the NKT agonist α-GalCer activate DN32.D3 NKT cells, demonstrating that breast cancer cells can directly interact with iNKT cells.
- Showed that exposure of DC to irradiated 4T1 cells and their lysates alters their phenotype, enhancing the expression of CD1d and CD86.
- Showed significant numbers of intratumoral CD11c dendritic cells in NKT-deficient mice when compared to wildtype BALB/c bearing subcutaneous tumors

REPORTABLE OUTCOMES

NATIONAL MEETINGS AND PRESENTATIONS

- Society for the Immunotherapy of Cancer, 25th Annual Meeting
  (formerly International Society for Biological Therapy of Cancer)
  2-4 Oct 2010 Washington DC
  Poster Presentation:
  Abstract # 71: ***Immunoregulation by Invariant Natural Killer T Cells in a Mouse Model of Metastatic Breast Cance***r
  Journal of Immunotherapy, 2010, Vol 33(8): 888.
  Pilones, KA, Kawashima, N, Babb, J, and Demaria, S.

- Radiation Research Society, 56th Annual Meeting
  26-29 Sept 2010 Maui, HI

  Posters:
  Abstract 2.58 : ***Inhibition of transforming growth factor beta radiosensitizes 4T1 murine mammary carcinoma in vitro and in vivo***
  Bouquet, SF, Pilones, KA, Demaria, S and Barcellos-Hoff MH.

  Abstract 7.74: ***Transforming growth factor beta inhibition uncovers radiation-induced anti-tumor immunity in a mouse breast cancer model.***
  Pilones, KA, Bouquet, SF, Demaria, S and Barcellos-Hoff MH

  Oral Presentation:
  Mini Symposium on Experimental Therapeutics and Translational Research

  Awards:
  Scholar-in-Training Travel Award

INSTITUTIONAL MEETINGS AND CONFERENCES

- NYU Immunology Club Journal club
  Meets every Tuesday

- NYU Cancer Institute Breast Biology Working Group
  Meets every 3rd Wednesday of every month

- NYU Dept of Pathology Works-in-Progress & Journal Club
  Meets every Tuesday 5PM

- STAR (Scientists-in-Training) Seminar Series
  Hosted by the NYU Postdoctoral Association
  March 17 2011

MENTORING:

- Sarah DiNapoli, BS
  Rotating student Sept 2010-present
  Accepted as a graduate student at Weill Cornell School of Graduate Biomedical Sciences

COLLABORATIONS:

- Mary Helen Barcellos-Hoff, PhD
  Department of Radiation Oncology, NYU School of Medicine

** We have also begun working closely with the lab of Dr Barcellos-Hoff on mechanisms of TGF-beta inhibition in cancer. Our collaboration has contributed significantly to our understanding of the immunosuppressive mechanisms within the tumor microenvironment. Given the pleiotropic role that TGFbeta plays in downregulating tumor immunosurveillance, it will be of great interest in this project to look at a possible link between TGFbeta and NKT-mediated immunoregulation. We have recently submitted a manuscript detailing our findings.*

CONCLUSIONS

Collectively, the in vitro data suggest that 4T1 cells are unlikely to present an endogenous lipid antigen to directly condition NKT cells to assume a regulatory function. Our data indicate, however, that a 4T1 tumor-derived lipid antigen can be loaded on CD1d molecules on the surface of dendritic cells and initiate NKT cell activation. Currently, studies are being done to characterize the nature of the NKT response that may hint a regulatory function. Since our data support a key role for dendritic cells in conditioning NKT cells, we will be conducting experiment to evaluate aspects of dendritic cell function (i.e, presentation, cross-priming) in greater detail. These experiments, as outlined in the approved statement of work, will comprise much of the work to be done in the second year.

In the last year, I have actively participated in departmental Works-in-Progress seminars and focused journal clubs (one in immunology and another in breast cancer research specifically) which have enriched my knowledge in cutting edge research in breast cancer. I have had the great opportunity to meet with leaders in the field of breast cancer immunology at recent meetings, and will continue to foster a collaborative relationship with them in the years to come. I continue to work closely with my mentor, Dr Sandra Demaria, who I meet with every week to discuss results and plan experiments. She continues to be an invaluable resource to my training as a future breast cancer scientist.

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APPENDICES

APPENDIX 1: Pilones, K.A., et al., ***Invariant natural killer T cells regulate breast cancer response to radiation and CTLA-4 blockade.*** Clinical Cancer Research, 2009. **15**(2): p. 597-606.

APPENDIX 2: Pilones, KA, et al ***Immunoregulation by Invariant Natural Killer T Cells in a Mouse Model of Metastatic Breast Cance***r Journal of Immunotherapy, 2010, Vol 33(8): 888

APPENDIX 1

Pilones, K.A., et al., ***Invariant natural killer T cells regulate breast cancer response to radiation and CTLA-4 blockade***.
Clinical Cancer Research, 2009. **15**(2): p. 597-606.

Invariant Natural Killer T Cells Regulate Breast Cancer Response to Radiation and CTLA-4 Blockade

Karsten A. Pilones,[1] Noriko Kawashima,[1] Anne Marie Yang,[1] James S. Babb,[2] Silvia C. Formenti,[3] and Sandra Demaria[1]

Abstract

Purpose: Immunoregulatory and suppressive mechanisms represent major obstacles to the success of immunotherapy in cancer patients. We have shown that the combination of radiotherapy to the primary tumor and CTL-associated protein 4 (CTLA-4) blockade induces antitumor immunity, inhibiting metastases and extending the survival of mice bearing the poorly immunogenic and highly metastatic 4T1 mammary carcinoma. Similarly to patients with metastatic cancer, however, mice were seldom cured. Here we tested the hypothesis that invariant natural killer T (iNKT) cells, a subset with unique regulatory functions, can regulate the response to radiotherapy and CTLA-4 blockade.

Experimental Design: The growth of 4T1 primary tumors and lung metastases was compared in wild-type and iNKT cell – deficient (iNKT-/-) mice. Treatment was started on day 13 when the primary tumors were palpable. Mice received radiotherapy to the primary tumor in two doses of 12 Gy in combination or not with 9H10 monoclonal antibody against CTLA-4. Response to treatment was assessed by measuring primary tumor growth delay/regression, survival, and number of lung metastases.

Results: The response to radiotherapy plus 9H10 was markedly enhanced in the absence of iNKT cells, with 50% of iNKT-/- versus 0% of wild-type mice showing complete tumor regression, long-term survival, and resistance to a challenge with 4T1 cells. Administration of the iNKT cell activator α-galactosylceramide did not enhance the response of wild-type mice to radiotherapy plus 9H10. Tumor-infiltrating iNKT cells were markedly reduced in wild-type mice treated with radiotherapy plus 9H10.

Conclusions: iNKT cells play a major role in regulating the response to treatment with local radiotherapy and CTLA-4 blockade.

Preclinical models and clinical trials have provided the proof of principle that immunotherapy can treat cancer (reviewed in ref. 1). However, despite the development of multiple vaccination strategies to induce antitumor T cells, objective responses are seen only in a small fraction of patients, and it remains unclear what factors determine the success of any given treatment. Increased understanding of the complex networks of immune cells and cytokines that control immune system function has led to the identification of several regulatory and suppressive mechanisms as major obstacles to the success of immunotherapy (2). These are of two types: those that are tumor-induced and those preexisting in the host as the result of genetic predisposition, age, concurrent diseases, or previous therapies (3). Myeloid-derived suppressor cells (MDSC; ref. 4) belong to the first category because their accumulation in tumor-bearing mice and cancer patients is driven by tumor growth (5). Regulatory T cells play a key role in the maintenance of self-tolerance as well as tolerance to tumors (6). Natural regulatory T cells develop in the thymus and belong to the second category (7). In contrast, adaptive regulatory T cells are generated in the periphery from mature T cells, and their differentiation is induced by the tumor microenvironment (8, 9).

Another T cell subset with regulatory function, natural killer T (NKT) cells, has been implicated in both up- and down-regulation of immune responses (10). NKT cells have unique properties in that they can rapidly produce both Th1 and Th2 cytokines upon activation, and function as a powerful switch to turn on or off the innate and adaptive immune response in various diseases and conditions (10 – 12). NKT cells recognize glycolipid antigens presented by CD1d molecules, and in mice most express a canonical α-chain (Vα14Jα18) and are known as invariant NKT (iNKT) or type I NKT cells. In humans, the homologous population of iNKT cells expresses Vα24 (10). iNKT cells react with α-galactosylceramide, a

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Metastatic breast cancer still represents a therapeutic challenge. Immunotherapy could offer an important alternative or adjunct to current treatments. Using as a model a known mouse tumor that behaves like aggressive metastatic breast cancer, we have previously shown that it is possible to successfully combine a standard treatment modality, local radiotherapy, with a novel antibody-based therapeutic agent that is currently being tested in clinical trials for cancer and targets the CTL-associated protein 4 (CTLA-4) receptor on T cells. The combination of local radiation to the primary tumor with CTLA-4–blocking antibodies elicited a CD8-mediated immune response inhibiting metastases and extending the survival of the animals. In preparation for the translation of this therapeutic approach to the clinic, we have investigated the regulatory mechanisms that determine the response to treatment. Data presented here indicate that invariant natural killer T (NKT) cells play a major role in down-regulating the response to radiation and CTLA-4 blockade in mice. This information will guide future studies in patients treated with a similar therapeutic strategy, and the potential clinical application of NKT cell–based immunotherapy against cancer.

synthetic CD1d-binding glycolipid that has a strong agonistic activity, whereas the noninvariant or type II NKT cells, although restricted by CD1d, do not bind α-galactosylceramide (13).

In cancer, NKT cells have been associated with spontaneous immunosurveillance in some experimental systems (14, 15), but have been shown to suppress immunosurveillance in others (16, 17). Recent work by Terabe et al. (18), suggests a functional difference between the two subsets of NKT cells in the regulation of antitumor immunity, with type II NKT cells being mostly responsible for immunosuppression. An immunoregulatory pathway that links the production of interleukin (IL)-13 by NKT cells to the induction of transforming growth factor β (TGFβ) production by MDSC has been shown to be responsible for the inhibition of antitumor CTL that results in tumor recurrence in some tumor models (16, 19, 20) but not in others (21, 22). Overall, the mechanisms of NKT cell–mediated immunoregulation in cancer involve multiple pathways and remain largely undefined.

Activation of iNKT cells by systemic administration of α-galactosylceramide to mice and humans has been shown to induce the rapid production of IFN-γ and IL-4 and other Th1 and Th2 cytokines (10). Importantly, in several preclinical cancer models this resulted in effective antitumor immunity mediated by the downstream activation of antigen-presenting cells, and NK and CD8-positive T cells (23). In initial clinical trials, however, α-galactosylceramide has not shown significant anticancer activity, possibly due to partially impaired NKT cell function in cancer patients (24).

Accumulated evidence in experimental models supports the development of a novel therapeutic approach based on the combination of ionizing radiation therapy with immunotherapy for the treatment of cancer (reviewed in refs. 25, 26). In most cases clinical trials testing the efficacy of novel treatments are done in patients with advanced-stage disease, a population for which the need for new therapies is most urgent, but that also presents with an immunologic environment largely altered by the tumor. To mimic this situation, we have employed as a model the poorly immunogenic 4T1 mouse mammary carcinoma. After s.c. inoculation 4T1 cells grow to form a highly invasive primary tumor that early on sheds spontaneous metastases to the lungs and other organs (27). Mice usually die of metastatic disease to the lungs. We previously tested the combination of radiotherapy with CTL-associated protein 4 (CTLA-4) blockade approximately two weeks after implantation in mice, when primary tumors are palpable and metastatic cells have already spread systemically (28). Whereas single modality treatment was ineffective, the combination of local radiotherapy to the primary tumor and CTLA-4 blockade elicited CD8 T-cell–dependent antitumor immunity. The immune response effectively inhibited the growth of spontaneous lung metastases, prolonging the survival time of animals. Cure was rare, however, and most mice eventually succumbed to their disease (28).

In this study we investigated whether the disruption of immunoregulatory circuits can improve the response to treatment with radiotherapy and CTLA-4 blockade. Our data indicate that whereas 4T1 tumors grew equally well in wild-type mice and mice lacking iNKT cells (iNKT-/-), the latter developed a CD8 response that partially inhibited metastases but not primary tumor growth. This observation is in agreement with a minor role of iNKT cells in regulating spontaneous immunosurveillance in the 4T1 model (18). Remarkably, as compared with wild-type mice, iNKT-/- mice showed a marked improvement in survival and cure rate following treatment with radiotherapy and CTLA-4 blockade, suggesting that iNKT cells can play a major role in regulating the response to treatment. Administration of α-galactosylceramide did not improve the response of wild-type mice to radiotherapy and CTLA-4 blockade. Despite the differential response to treatment, wild-type and iNKT-/- mice showed a similar systemic and intratumoral increase in MDSC around the time treatment was started, suggesting that the degree of MDSC accumulation does not predict the response to treatment.

Materials and Methods

Mice. Six- to eight-week-old BALB/c mice were obtained from Taconic Animal Laboratory. iNKT-/- (Vα14 Jα18-deficient) mice (29) in the BALB/c background obtained from M. Taniguchi (RIKEN Research Center for Allergy and Immunology) were bred at New York University and used between 6 and 12 wk of age. All experiments were approved by the Institutional Animal Care and Use Committee of New York University.

Cells and reagents. 4T1 is a BALB/c mouse–derived mammary carcinoma cell line (provided by Fred Miller, the Michigan Cancer Center; ref. 27) and A20 is a BALB/C mouse–derived B-cell leukemia/lymphoma (30). 4T1 cells were grown in DMEM (Invitrogen Corporation) supplemented with 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 2.5×10^{-5} mol/L 2-mercapthoethanol, and 10% fetal bovine serum (Gemini Bio-Products; complete medium). These cells were found to be free of contamination by mycoplasma by the Mycoplasma detection kit (Roche Diagnostics). Anti-CTLA-4 hamster monoclonal antibody (mAb) 9H10 was purified as previously described (28). Control hamster IgG was purchased from Jackson Immunoresearch Laboratories. Purified anti-CD4 (GK1.5), anti-CD8 (2.43) rat mAb and control rat IgG were purchased from BioExpress, Inc.

Tumor challenge and treatment. The mice were injected s.c. in the right flank with 5×10^4 4T1 cells in 0.1 mL of DMEM without additives on day 0. Perpendicular tumor diameters were measured with a Vernier caliper, and tumor volumes were calculated as length $\times$ width^2 $\times$ 0.52. On day 13, when tumors reached the average diameter of 5 mm (approximately 65 mm^3 in volume) animals were randomly assigned to various treatment groups, as indicated. Radiotherapy was administered as previously described (28). Briefly, all mice (including mice receiving mock radiation) were lightly anesthetized by i.p. injection of avertin (240 mg/kg) then positioned on a dedicated plexiglass tray, and the whole body was protected by lead shielding, except for the area of the tumor to be irradiated. Radiotherapy was delivered to a field including the tumor with 5-mm margins using a ^{60}CO radiation source by two fractions of 12 Gy each on days 13 and 14. Control hamster IgG and 9H10 were given i.p. at 200 μg at 1, 4, and 7 d after radiotherapy. α-Galactosylceramide (Alexis Biochemicals) was dissolved in DMSO and diluted in PBS supplemented with 0.5% polysorbate-20 (w/v) prior to injection and given i.p. at 100 ng/mouse twice a week, starting on day 1 postradiotherapy. Tumor growth was evaluated every 2 to 3 d until death or sacrifice when tumor dimensions exceeded 5% body weight or mice showed dyspnea, abnormal posture, >20% body weight loss, difficulty with ambulation, or any other clinical sign of metastatic disease causing significant pain or distress, according to institutional guidelines. In some experiments, mice that rejected the tumors and survived tumor-free until day 120 were injected in the controlateral flank with a tumorigenic inoculum of either 4T1 or A20 cells and followed for up to 60 d for tumor development.

Clonogenic lung metastases assay. For determination of the number of clonogenic lung metastases, lungs were harvested on day 33 or 35 post–s.c. injection in the right flank with 5×10^4 4T1 cells and processed as previously described (27). Briefly, lungs from each individual animal were minced into 1-mm pieces, and digested with 5 mL enzyme cocktail containing 1 mg/mL collagenase IV and 6 units/mL elastase, both from Sigma Chemical Company, in PBS for 1 h at 4°C with rotation. Cell suspensions were filtered through 70-μm nylon cell strainers and washed two times with HBSS, then resuspended in complete medium. Serial 3- to 5-fold dilutions were plated in 10-cm tissue culture dishes in the presence of 60 μmol/L 6-thioguanine (2-amino-6-mercaptopurine; Sigma Chemical Company) to allow only the growth of 4T1 cells which are resistant to this drug (27). When colonies of growing 4T1 cells became visible (8-14 d) the plates were washed with PBS, fixed with methanol, and stained with Crystal violet. The colonies were counted independently by two to three investigators, blinded to the group to which each mouse belonged, and the total number/lungs was calculated for each animal.

In vivo *T-cell subset depletion.* Depletion of CD4 and CD8 T-cell subsets was done by injecting GK1.5 or 2.43 mAb i.p. at 100 μg/mouse on 3 consecutive d, starting 1 d before the s.c. injection in the right flank with 5×10^4 4T1 cells. The depletion was maintained by repeated weekly injections of mAb. Depletion was confirmed by testing spleen cells from control animals for the presence of CD4 and CD8 T-cells using non–cross-reactive FITC-RMA4-4 and PE-anti-CD8β mAb (BD PharMingen).

Mice vaccination. Wild-type and iNKT-/- mice were vaccinated with 10^6 irradiated (100 Gy) 4T1 cells s.c. in the right flank 3 times at weekly intervals. Control mice received DMEM. Seven days after the last vaccination the mice were challenged with a tumorigenic inoculum (5×10^4) of 4T1 cells s.c. in the opposite flank and followed for tumor development.

Analysis of MDSC and iNKT cells infiltrating 4T1 tumors. The tumors were dissected carefully removing all surrounding normal tissue, minced into 1-mm pieces, and digested with collagenase D (400 U/mL) for 25 min at 37°C in a shaker. Ten milliliters of the enzyme solution were used for every gram of tumor tissue. Obtained cell suspensions were filtered through 40-μm nylon cell strainers and washed two times with HBSS. Aliquots of 10^6 tumor-derived cells were incubated with antimouse CD16/32 (Fc block) for 10 min followed by staining at 4°C with mAb against mouse Gr-1-Cy-Chrome and CD11b-PE (BD PharMingen) to identify MDSC, or with PE-conjugated mCD1d/PBS57 tetramer (provided by the NIH Tetramer Facility, ref. 31) and FITC-conjugated CD3 to identify iNKT cells. Unloaded PE-conjugated mCD1d tetramer was used as control. Samples were analyzed using a FACScan flow cytometer and FlowJo version 6.4.4 (Tree Star).

Measurement of TGF β1 production. 1×10^6 spleen cells from individual mice were cultured o.n. in RPMI 1640 medium supplemented with 1% fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin in a 24-well plate. Supernatants were harvested and stored at -80°C. The concentration of TGF β1 was determined in duplicate samples using the Quantikine TGF β1 Immunoassay Kit (R&D Systems) following the manufacturer's instructions. Background readings for culture medium were subtracted from all samples. As previously reported (19), acidification of the samples was required to detect the latent form of TGF β1 produced in vitro by MDSC.

Immunostaining of tumor sections. 4T1 tumors from treated and untreated wild-type and iNKT-/- mice were harvested on day 29 posttumor inoculation, fixed for 1 h at 4°C in 4% paraformaldehyde followed by overnight incubation in 30% sucrose, and frozen in optimum cutting temperature medium. Sections (8 μm) were incubated with 0.1% Tween-20 and 0.01% Triton-X100 for 20 min, followed by 4% rat serum in 4% bovine serum albumin/PBS for an additional 30 min. Sections were stained with PE-Texas-Red-conjugated rat antimouse CD4 or PE-conjugated rat antimouse CD8α (Caltag), and counterstained with 5 μg/mL 4′, 6-diamidino-2-phenylindole (Sigma). Images were obtained using a Nikon Eclipse 800 deconvolution microscope. The CD4 and CD8 T cells were counted in three randomly selected (20×) fields in each tumor.

Statistical analysis. ANOVA based on ranks was used to assess differences among animal groups defined by genotype and/or treatment received with respect to tumor weight, tumor volume, or the number of lung metastases at a fixed time point. Specifically, each end point (tumor weight, tumor volume, number of metastases) was first converted to ranks within each experiment and the ranks were then used as the dependent variable in the analysis of variance. Ranks were used in place of the observed values to better satisfy underlying distributional assumptions. When the treatment groups constituted a 2 × 2 factorial design (presence/absence of CTLA-4 blockade/radiotherapy), the analysis examined the main effects for each treatment modality (radiotherapy, CTLA-4 blockade) and the interaction between the modalities. The log-rank test was used to compare animal groups in terms of overall survival, defined as time to death or sacrifice. The median and mean survival times within each treatment arm were estimated using the Kaplan-Meier product-limit method, and a 95% confidence interval for each median survival time was derived on the basis of a sign test. All reported P values are two-sided and were declared statistically significant at the 5% level. The statistical computations were carried out using SAS for Windows, version 9.0 (SAS Institute).

Results

iNKT-/- mice develop a spontaneous CD8-dependent antitumor immune response inhibiting metastases. To investigate the role of the host immune status in tumor growth and response to treatment we injected mice lacking iNKT cells with 4T1 cancer cells. Whereas primary tumors grew at the same rate in iNKT-/- and wild-type mice (not shown) and primary tumor weight was not significantly different on day 34 (mean ± SD, 1,364 ± 895 and 873 ± 234 mg for iNKT-/- and wild-type mice, respectively; $P = 0.41$), the number of metastatic cells in the lungs was significantly lower in iNKT-/- mice (mean ± SD, 525.4 ± 1461.9 and1,364.0 ± 895.5 for iNKT-/- and wild-type, respectively; $P = 0.0009$; Fig. 1A). The depletion of CD8 T cells or CD4 and CD8 T cells abrogated the difference in lung

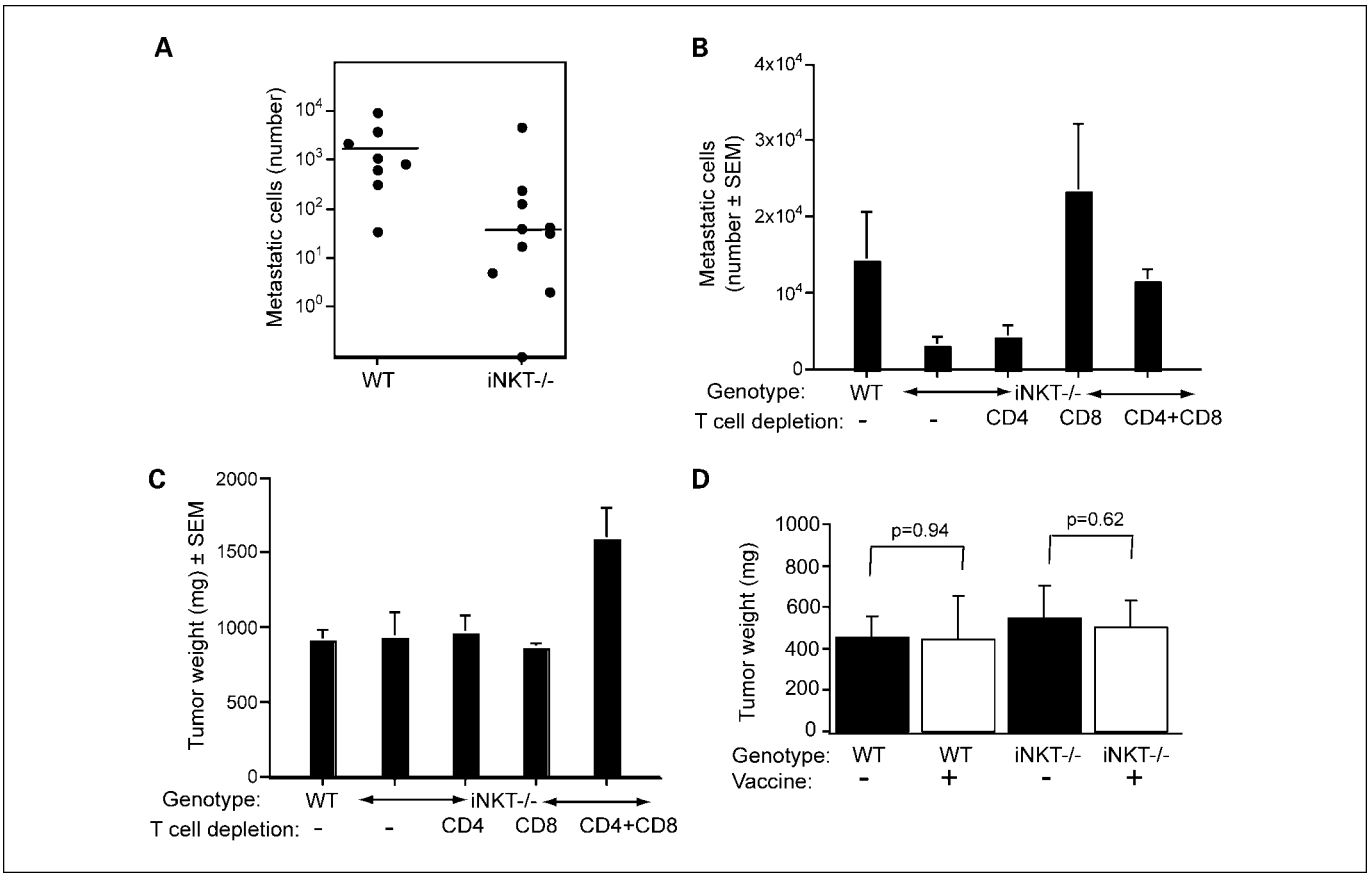

Fig. 1. CD8 T cell – mediated inhibition of metastases in untreated tumor-bearing mice lacking iNKT cells. *A,* mice received 5×10^4 4T1 cells s.c. on day 0. The number of metastatic cells in the lungs on day 34 was significantly lower for the iNKT-/- than for the wild-type mice ($P = 0.0009$). Each symbol represents one animal. *B, C,* mAb-mediated CD4 and CD8 T cell depletion was started on day-1, and maintained up to the day of sacrifice. Mice were sacrificed on day 33 to determine the number of metastatic cells in the lungs (*B*) and tumor weight (*C*). Bars, mean ± SE of 8 to 10 animals/group. *B,* the number of metastases in iNKT-/- mice depleted of CD8 T cells (*CD8*) or CD4 and CD8 T cells (*CD4+CD8*) was significantly higher than in nondepleted iNKT-/- mice ($P = 0.004$ and 0.001, respectively). In contrast, CD4 depletion did not significantly increase the number of metastases ($P = 0.94$). Whereas there was a significant difference between wild-type and nondepleted iNKT-/- mice in the number of metastases ($P = 0.043$), no significant difference was seen between wild-type and iNKT-/- mice after CD8 T cell depletion by itself or in combination with CD4 T cell depletion. *C,* there was no significant tumor weight difference ($P > 0.6$) between wild-type and iNKT-/- mice depleted or not depleted of CD8 or CD4 T cells. However, tumor weight was significantly increased in mice depleted of both T cell subsets ($P < 0.01$ when compared with all other groups). *D,* 4T1 cells are not intrinsically immunogenic in iNKT-/- mice. Wild-type and iNKT-/- mice ($n = 5$/group) were vaccinated weekly for 3 wk with 10^6 irradiated (100 Gy) 4T1 cells s.c. in the left flank (+) or mock vaccinated with DMEM (-). Seven days after the last vaccination mice were challenged with 5×10^4 viable 4T1 cells and followed for tumor development. All mice developed tumors and there was significant difference in tumor weight in vaccinated wild-type ($P = 0.94$) or iNKT-/- ($P = 0.62$) mice on day 26.

metastases between wild-type and iNKT-/- mice, whereas the depletion of CD4 T cells did not have any effect, indicating that CD8 T cells are required for the inhibition of lung metastases observed in iNKT-/- mice (Fig. 1B). Interestingly, primary tumor growth was not affected by depletion of CD8 or CD4 T cells but a statistically significant increase in tumor weight ($P < 0.01$) was seen in double-depleted mice (Fig. 1C).

The spontaneous development of antitumor CD8 T cells could be due to increased intrinsic immunogenicity of 4T1 cells in iNKT-/- mice. To test this hypothesis, wild-type and iNKT-/- mice were immunized repeatedly with 4T1 cells inactivated by irradiation, followed 7 days after the last immunization by challenge with 5×10^4 live 4T1 cells. If antitumor CD8 cells had developed following vaccination, they would prevent or inhibit growth of the early small tumors. In contrast, all wild-type and iNKT-/- mice developed tumors, and there was no significant difference in tumor weight between 4T1-vaccinated and nonvaccinated mice (Fig. 1D). Therefore, 4T1 cells do not show increased immunogenicity in iNKT-/- mice.

In the absence of iNKT cells, mice bearing established 4T1 mammary carcinoma show a markedly enhanced therapeutic response to treatment with local radiotherapy and CTLA-4 blockade. We have shown that wild-type mice bearing the poorly immunogenic 4T1 mammary carcinoma develop antitumor CD8 T cells inhibiting spontaneous lung metastases and extending their survival when treated with the combination of local radiotherapy to the primary tumor and CTLA-4 – blocking mAb 9H10, whereas each single modality did not have an effect on survival (28). However, complete cure of mice with well-established disease remained rare: 0% to 15% of the mice were cured in different experiments (28).[4] This suggested that the duration and/or potency of the antitumor immune response elicited by treatment was limited in most animals. To test if iNKT cells play a role in response to treatment, iNKT-/- mice with established 4T1 tumors were randomly assigned to be treated with radiotherapy,

[4] Our unpublished results.

CTLA-4 blockade, or a combination of the two modalities. In the absence of treatment, tumors grew progressively, and all mice were dead by day 49. Radiotherapy alone was able to cause a significant ($P < 0.05$) growth delay of the irradiated tumor, and complete regression in 2 of 9 mice. Despite the fact that the growth of all irradiated tumors was delayed, there was no statistically significant improvement in survival ($P = 0.16$; Fig. 2A and B). This is consistent with the fact that radiotherapy as a single modality cannot significantly inhibit the lung metastases outside of the field of radiation and, therefore, cannot extend survival, as previously observed in wild-type mice (28). However, the two iNKT-/- mice treated with radiotherapy alone that showed complete regression of the primary tumor remained tumor-free on day 120, and showed the development of a protective antitumor response, as described below.

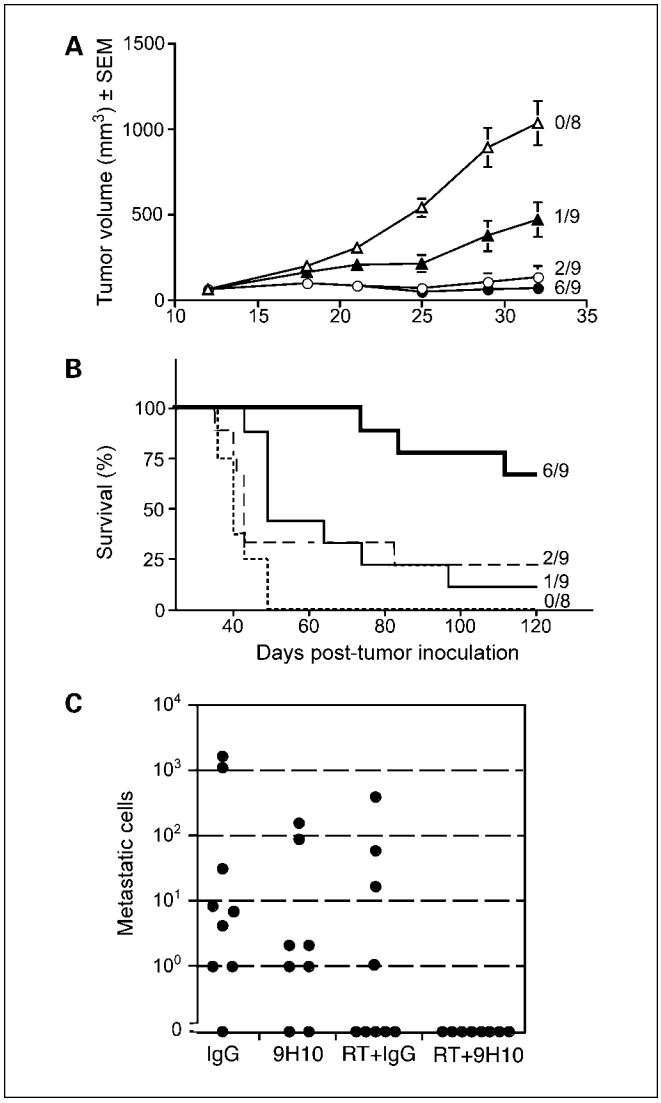

Fig. 2. Therapeutic response of iNKT-/- mice with established 4T1 carcinoma to treatment with local radiotherapy and CTLA-4 blockade. Treatment was started on day 13 post – s.c. inoculation with 4T1 cells. Radiotherapy (*RT*) was delivered in two fractions of 12 Gy to the s.c. tumors on days 13 and 14. Antibodies were given i.p. 1, 4, and 7 d postradiotherapy. *A,* tumor growth delay in iNKT-/- mice treated with control hamster IgG (IgG, *open traingles*; $n = 8$), 9H10 (*closed triangles*; $n = 9$), radiotherapy plus IgG (*open circles*; $n = 9$), or radiotherapy plus 9H10 (*closed circles*; $n = 9$). Tumor volume is shown as the mean ± SE for animals with tumors in each treatment group up to day 32 when all animals were alive. The number of mice with complete tumor regression over the total number of mice per group is indicated. Tumor volume differences between treated and control (IgG) mice were statistically significant ($P < 0.05$) from day 18 for the radiotherapy and the radiotherapy plus 9H10 groups, and from day 21 for the 9H10 group. *B,* percentage of surviving mice following treatment with control hamster IgG (IgG; *broken line*; $n = 8$), 9H10 (*thin line*; $n = 9$), radiotherapy plus IgG (*dashed line*; $n = 9$), or radiotherapy plus 9H10 (*bold line*; $n = 9$). The number of mice surviving and tumor-free up to day 121 over the total number of mice per group is indicated. Treatment with radiotherapy plus 9H10 ($P < 0.0001$) and 9H10 ($P = 0.002$) resulted in a statistically significant improvement in overall survival as compared with control (IgG) mice, whereas treatment with radiotherapy alone did not ($P = 0.16$). *C,* number of metastatic cells in the lungs of iNKT-/- mice at day 35 post – s.c. tumor inoculation. Each symbol represents a single animal. Treatment groups are as above. Relative to control (IgG) mice, the number of metastases was significantly lower in the radiotherapy plus 9H10 ($P = 0.004$) group but not in the radiotherapy plus IgG ($P = 0.15$) and 9H10 ($P = 0.3$) groups.

iNKT-/- mice receiving CTLA-4 – blocking mAb as a single modality showed a significant ($P < 0.05$ from day 21 compared with control), although less pronounced than that obtained with radiotherapy, tumor growth delay, and the tumor regressed completely in 1 of 9 mice (Fig. 2A). This animal was cured of tumor, whereas the rest of the group showed a significant ($P = 0.002$) but relatively modest increase in survival when compared with the control group (Fig. 2B). This differs from results obtained in wild-type mice in which CTLA-4 blockade did not have any effect on primary tumor growth or survival (28).

Treatment with the combination of radiotherapy and CTLA-4 blockade caused complete regression of the irradiated tumor in 6 of 9 mice (Fig. 2A and B), and a marked extension of their survival with all animals alive on day 74 ($P < 0.0001$ compared with control group). All tumor-free mice that survived until day 120 were considered "cured" and were rechallenged with a tumorigenic inoculum of 4T1 cells (two mice in radiotherapy, one in 9H10, and three in radiotherapy plus 9H10 group) or the syngeneic but unrelated A20 lymphoma cells (three mice in radiotherapy plus 9H10 group). All 6 mice rejected the 4T1 tumor, whereas 5 of 5 naïve mice challenged at the same time developed tumors. In contrast, the A20 tumor grew in the three survivor mice and three naïve mice (data not shown). These data indicate that iNKT-/- mice cured of tumor developed a long-lasting tumor-specific memory response.

To directly determine the effect of treatment on lung metastases, another experiment was done in which tumor-bearing mice were treated as above and lungs analyzed on day 35. Although single treatment with either radiotherapy or 9H10 showed a tendency to lower the number of metastatic cells, the difference was not statistically significant compared with control IgG-treated mice (Fig. 2C). In contrast, mice treated with radiotherapy and CTLA-4 blockade showed complete inhibition of lung metastases (Fig. 2C). The observation that the effect of radiotherapy on lung metastases was significant only in the presence of 9H10 is consistent with a synergism between radiotherapy and CTLA-4 blockade.

Next, responses to the combination treatment with radiotherapy and CTLA-4 blockade were directly compared between 4T1 tumor – bearing wild-type and iNKT-/- mice. In the absence of treatment, there was no difference in primary tumor growth between wild-type and iNKT-/- mice (Fig. 3A). However, untreated iNKT-/- mice showed a small but statistically significant increase in survival ($P = 0.008$; mean, 43.8 and 37.1 days for iNKT-/- and wild-type mice, respectively). This is consistent with the CD8-mediated inhibition of metastases in iNKT-/- mice (Fig. 1).

Treatment with radiotherapy and CTLA-4 blockade initially caused a significant primary tumor growth delay in both wild-type and iNKT-/- mice, but only iNKT-/- mice went on to

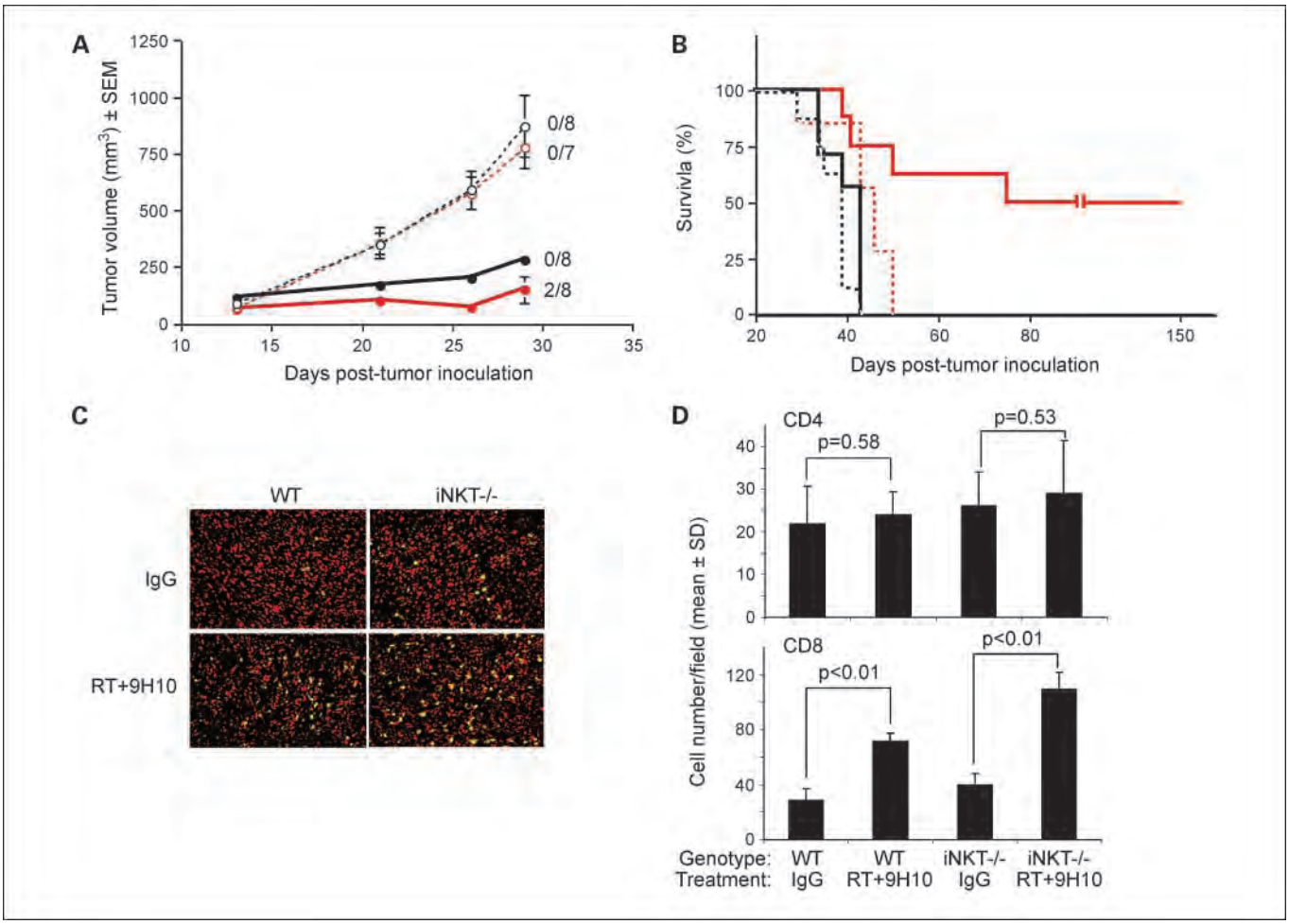

Fig. 3. Comparison between wild-type and iNKT-/- mice with established 4T1 carcinoma in the response to treatment with local radiotherapy and CTLA-4 blockade. Comparison of response to treatment with radiotherapy and 9H10 (*solid lines*) or IgG (*broken lines*) in wild-type (*black*) and iNKT-/- (*red*) 4T1 tumor – bearing mice. Treatment was started on day 13 post – s.c. inoculation in the flank. Radiotherapy was delivered in two fractions of 12 Gy to the s.c. tumors on days 13 and 14. Antibodies were given i.p. 1, 4, and 7 d postradiotherapy. *A,* tumor volume is shown as the mean ± SE for animals with tumors in each treatment group up to day 29 when all animals were alive. The number of mice with complete tumor regression by day 29 over the total number of mice per group is indicated. Two additional iNKT-/- mice had complete tumor regression after day 29. Primary tumor growth was not significantly different in wild-type and iNKT-/- mice receiving the control IgG ($P > 0.05$) at all time points. In contrast, iNKT-/- mice receiving radiotherapy plus 9H10 had a significantly lower tumor volume than wild-type mice receiving radiotherapy plus 9H10 ($P < 0.05$) from day 21. Tumor volume differences between radiotherapy plus 9H10 and control (IgG) mice were statistically significant ($P < 0.05$) from day 21 within each genotype. *B,* data are shown as the percentage of surviving mice over time. Surviving tumor-free mice were followed up to day 150. iNKT-/- mice treated with radiotherapy plus 9H10 showed a statistically significant improvement in overall survival ($P < 0.05$) as compared with all other groups. Half of the iNKT-/- mice but none of the wild-type mice were cured from established 4T1 tumors by treatment with radiotherapy and CTLA-4 blockade. *C, D,* in a separate group of mice treated as above, tumors were excised at day 29 and analyzed by fluorescence microscopy for the presence of CD4-positive and CD8-positive T cells. *C,* representative fields showing CD8-positive T cells (*green*) infiltrating 4T1 tumors in wild-type and iNKT-/- mice treated as indicated. Nuclei were stained with 4′, 6-diamidino-2-phenylindole (*red*). *D,* mean numbers of CD4-positive and CD8-positive tumor-infiltrating lymphcytes in three mice per group. In both wild-type and iNKT-/- mice, CD8-positive but not CD4-positive tumor-infiltrating lymphcytes were increased by radiotherapy plus 9H10 treatment, but the increase was more pronounced in iNKT-/- mice ($P = 0.006$).

achieve complete tumor regression and cure, as shown by the absence of tumor on day 150 in 50% of the animals (Fig. 3A and B). None of the wild-type mice survived long-term, suggesting that only in iNKT-/- mice was the duration and potency of the antitumor immune response sufficient to completely eradicate the tumor.

Analysis of tumor-infiltrating lymphocytes on day 29 showed that CD4-positive cells were present in similar quantities in 4T1 tumors growing in wild-type and iNKT-/- mice, and were not significantly increased by treatment with radiotherapy and CTLA-4 blockade (Fig. 3C). In contrast, following treatment there was a significant increase in the number of CD8-positive cells infiltrating 4T1 tumors in both wild-type and iNKT-/- mice ($P < 0.01$ in treated versus untreated mice of both genotype; Fig. 3D). Importantly, the number of CD8-positive tumor-infiltrating lymphocytes was higher in iNKT-/- mice than in wild-type mice, and this difference was highly significant following treatment ($P = 0.006$ in treated iNKT-/- versus treated wild-type mice). These data confirm our previous observations that CD8-positive but not CD4-positive T cells are responsible for 4T1 tumor inhibition induced by treatment with radiotherapy and CTLA-4 blockade (28, 32). In addition, they suggest that the antitumor response induced by treatment is stronger in the absence of iNKT cells.

The iNKT cell-specific activator α-galactosylceramide does not improve the response of tumor-bearing wild-type mice to treatment with radiotherapy and CTLA-4 blockade. α-Galactosylceramide, a potent activator of iNKT cells, has been shown to induce powerful antitumor immune responses in several tumor models (23). Moreover, in a burn injury model, administration

of α-galactosylceramide was able to prevent the immunosuppression that is mediated by iNKT cells (33). To determine whether administration of α-galactosylceramide to 4T1 tumor–bearing mice could activate iNKT cells to carry out stimulatory rather than inhibitory functions, and improve the response of wild-type mice to radiotherapy and CTLA-4 blockade, mice were given α-galactosylceramide starting one day postradiotherapy. As a single modality α-galactosylceramide did not have any effect on tumor growth, and it did not improve the tumor growth delay or survival caused by treatment with radiotherapy and radiotherapy plus 9H10 (Fig. 4 and data not shown). However, a modest but statistically significant tumor growth delay was seen in mice treated with α-galactosylceramide and 9H10 ($P = 0.0396$ compared with control vehicle–treated mice; Fig. 4).

Overall, these data indicate that administration of α-galactosylceramide to wild-type mice with established tumors does not improve their response to treatment with radiotherapy and CTLA-4 blockade.

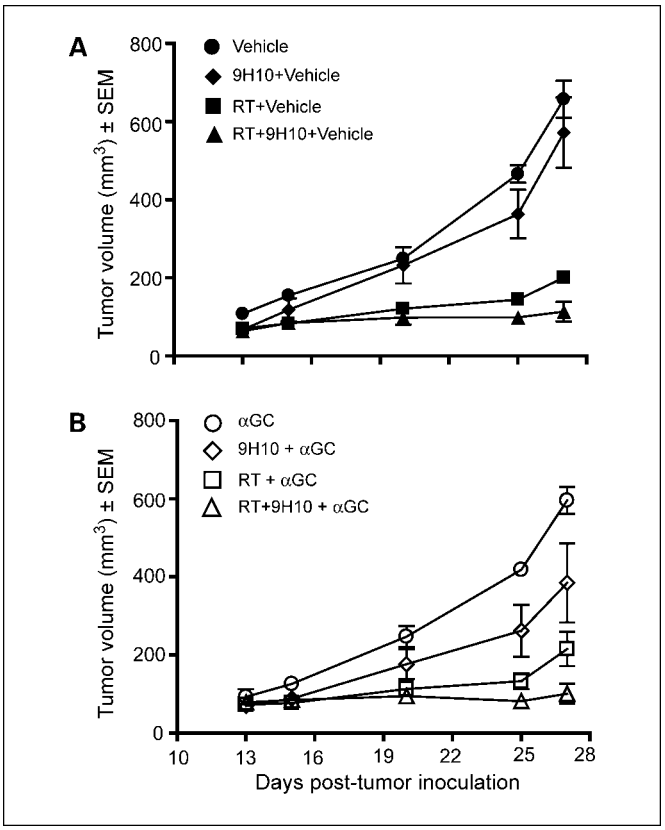

Fig. 4. Administration of α-galactosyleramide does not enhance the response of 4T1 tumor–bearing wild-type mice to local radiotherapy and CTLA-4 blockade. Treatment was started on day 13 post–s.c. inoculation in the flank. Radiotherapy was delivered in two fractions of 12 Gy to the s.c. tumors on day 13 and 14. 9H10 was given i.p. 1, 4, and 7 d postradiotherapy. α-Galactosyleramide (100 ng) was given i.p. twice per week starting on day 1 postradiotherapy. Wild-type mice ($n = 5$/group) were treated with (*A*) vehicle (*closed circles*), 9H10 plus vehicle (*closed diamonds*), radiotherapy plus vehicle (*closed squares*), radiotherapy plus 9H10 plus vehicle (*closed triangles*), (*B*) α-galactosyleramide (*open circles*), 9H10 plus α-galactosyleramide (*open diamonds*), radiotherapy plus α-galactosyleramide (*open squares*), or radiotherapy plus 9H10 plus α-galactosyleramide (*open triangles*). Tumor volume is shown as the mean ± SE for animals with tumors in each treatment group up to day 27 when all animals were alive. Radiotherapy caused a significant ($P < 0.001$ compared with control) tumor growth delay that was slightly enhanced by 9H10 but not by α-galactosyleramide. Although tumor volume differences between mice treated with α-galactosyleramide plus 9H10 and control were statistically significant ($P = 0.0396$), the triple combination of α-galactosyleramide, radiotherapy, and 9H10 did not show better tumor control than the combination of radiotherapy plus 9H10, and no complete tumor regression was observed.

Reduction in tumor-infiltrating iNKT cells after treatment with radiotherapy and CTLA-4 blockade. The presence within tumors of T cells with regulatory function has been shown to play an important role in the suppression of antitumor immunity (8). To determine whether iNKT cells were present within 4T1 tumors growing in wild-type mice, cell suspensions prepared from 4T1 tumors and lungs were stained with CD3 and CD1d tetramers loaded with the α-galactosylceramide analog PBS-57 (31). In untreated mice iNKT cells represented ~4% of T cells infiltrating 4T1 tumors and 3% of T cells isolated from the lungs (Fig. 5A and B). Importantly, there was a pronounced decline in iNKT cells in both primary tumor and lungs following treatment with radiotherapy plus 9H10 (Fig. 5B and C). Overall, these data indicate that iNKT cells are recruited to "primary" 4T1 tumors and their metastases, and that a treatment stimulating antitumor immunity leads to a relative decline in iNKT cell numbers, an observation that supports a regulatory role for iNKT cells.

Wild-type and iNKT-/- mice do not differ in the tumor-driven expansion and recruitment of MDSC to tumors. Suppression of antitumor immune responses by NKT cells has been linked to their ability to induce TGFβ production by MDSC (19). To determine whether the markedly increased response to treatment in iNKT-/- mice was due to decreased accumulation of MDSC, the numbers of MDSC in the spleen and primary tumors of wild-type and iNKT-/- mice were analyzed on day 14 of tumor growth, corresponding approximately to the time therapy was initiated. MDSC accumulation in spleens as well as primary tumors was similar between wild-type and iNKT-/- mice (Fig. 6A and B). Next, spleen cells of tumor-bearing wild-type and iNKT-/- mice were tested for secretion of TGFβ in short-term cultures. As previously reported for the 15-12RM tumor model (19), the secretion of TGFβ by spleen cells from 4T1 tumor–bearing wild-type mice was significantly increased compared with healthy mice ($P < 0.05$). This was true also for cells derived from iNKT-/- mice (Fig. 6C). Although the baseline production of TGFβ did not differ significantly between wild-type and iNKT-/- healthy mice ($P = 0.35$), tumor-bearing iNKT-/- mice produced more TGFβ than tumor-bearing wild-type mice, indicating that the production of TGFβ was not dependent on the presence of iNKT cells. Overall, these results are consistent with a recent report showing that NKT cells are not required to activate MDSC (34). They also indicate that the improved response to treatment with radiotherapy and CTLA-4 blockade seen in tumor-bearing iNKT-/- mice cannot be explained by differences in MDSC numbers or functional activation to produce TGFβ.

Discussion

In this study, we show that mice lacking the iNKT cell subset and bearing well-established 4T1 tumors respond to treatment with the combination of local radiotherapy and CTLA-4 blockade with markedly increased overall survival and cure rate as compared with wild-type mice (Figs. 2 and 3 and ref. 28). The improved response to treatment was not a result of increased immunogenicity of 4T1 cells in iNKT-/- mice because the growth of 4T1 tumors was similar in wild-type and iNKT-/- mice and vaccination with irradiated tumor cells did not induce

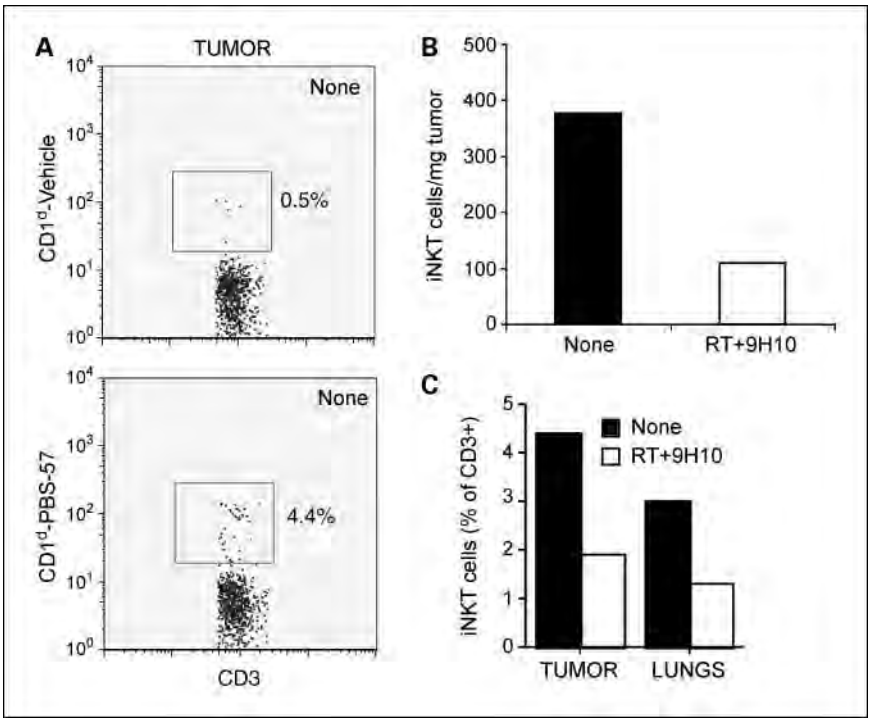

Fig. 5. iNKT cells infiltrating 4T1 tumors are reduced by treatment with local radiotherapy and CTLA-4 blockade. Wild-type mice were injected with 4T1 cells on day 0 and left untreated (None) or treated with local radiotherapy and 9H10 (radiotherapy plus 9H10) as described in the legend of Fig. 3. Tumors and lungs were harvested on day 29, and obtained single cell suspensions stained with FITC-anti-CD3 mAb and CD1d-PBS-57 PE or control CD1d-Vehicle PE tetramers to identify iNKT cells, followed by flow cytometry analysis. The lymphocyte gate was set based on the scattered plots in the spleen. *A,* histograms showing tumor-derived cells from untreated mice stained with CD3 and CD1d-PBS-57 or control tetramers, as indicated, and gated on CD3-positive cells. Numbers indicate the percentage of cells in the square gate. *B,* the percentage of cells in the lymphocyte gate positive for CD3 and CD1d-PBS-57 was multiplied for the percentage of cells in the lymphocyte gate and for the total number of viable cells isolated from the tumors, and divided for the tumor weight to obtain the number of cells per milligram of tumor in treated (radiotherapy plus 9H10, *white bar*) and untreated (None, *black bar*) mice. *C,* percentage of CD3-positive T cells that bind the CD1d-PBS-57 tetramers in tumor and lungs of treated (radiotherapy plus 9H10, *white bars*) and untreated (None, *black bars*) mice, as indicated. All data are from 4 to 5 mice of each group. Errors bars are absent because pooling of tumors and lungs within each group was necessary to obtain sufficient cells for analysis.

a protective antitumor response (Fig. 1D). However, iNKT-/- mice developed a spontaneous CD8 response inhibiting lung metastases (Fig. 1).

The development of an antitumor immune response that is at least partially effective against metastases in face of a poorly immunogenic growing primary tumor is an example of concomitant tumor immunity (35). This well-known phenomenon is consistent with the notion that invasive cancers cause tissue damage and the generation of inflammatory signals attracting innate immune cells and eventually leading to the activation of the adaptive immune system (36). Regulatory T cells have been shown to suppress concomitant immunity to the poorly immunogenic B16 melanoma (37). Our data suggest that iNKT cells play a similar role in the case of the poorly immunogenic 4T1 carcinoma and are consistent with a previous observation that iNKT-/- mice have improved immunosurveillance against 4T1 metastases compared with wild-type mice (18). Importantly, whereas Terabi et al. (18) detected the immune response against metastases after surgical removal of the primary tumor, our work provides the evidence that it is generated in the absence of any surgical manipulation that could potentially contribute to the development of the antitumor response (38).

Treatment of iNKT-/- mice with local radiotherapy used as a single modality caused a reduction in lung metastases that, however, was not statistically significant (Fig. 2C). Consistent with this, despite the complete "cure" obtained in ~20% of the mice, the effect of radiotherapy alone on survival was not significant ($P = 0.16$; Fig. 1B). This suggests that radiotherapy by itself was either not sufficient to induce a T cell response against this poorly immunogenic tumor, or that if induced it was suppressed (39). However, in combination with CTLA-4 blockade there was a highly significant antitumor effect leading to complete clearance of primary tumor and metastatic disease in over half of the mice (Figs. 2 and 3). Overall, these data are consistent with the hypothesis that radiotherapy causes changes in the tumor microenvironment that can promote the afferent and efferent phases of the antitumor immune response (26, 32, 40).

CTLA-4 blockade used as a single modality had a modest but detectable effect in inhibiting tumor growth and extending the survival of iNKT-/- but not wild-type mice (Fig. 2 and ref. 28). This result was confirmed in two additional experiments (data not shown). CTLA-4 blockade used as a single treatment is known to be effective only against more immunogenic tumors in wild-type mice (41). This suggests that besides the intrinsic immunogenicity of a tumor, preexisting host-specific factors can determine the response to CTLA-4 blockade. It is intriguing to consider whether the number or activity of iNKT cells could be a determinant of therapeutic responses mediated by anti-CTLA-4 antibodies in cancer patients (42).

Immunosuppression by NKT cells has been previously shown to be mediated by MDSC and TGFβ in some tumor models (16, 19, 20). In the 4T1 model, the increase in splenic MDSC in tumor-bearing mice was shown to correlate with the suppression of the immune response against metastases (43). Given the development of a spontaneous CD8 response inhibiting metastases in iNKT-/- mice, we expected to find less MDSC in iNKT-/- than in wild-type mice. In contrast, neither MDSC nor TGFβ production was reduced in the spleen of iNKT-/- mice (Fig. 6). This suggests that MDSC likely exert their suppression locally, within the growing primary tumors, as previously shown by Kusmartsev et al. (44). In mice treated with radiotherapy, sensitization of CD11b-positive MDSC by tumor-derived antigens that are released by radiation (40) could promote their destruction by antitumor CTL activated by anti-CTLA-4 antibodies leading to complete regression of established primary tumors.

Administration of the potent iNKT cell activator α-galactosylceramide did not improve the response of wild-type mice to

treatment with local radiotherapy and CTLA-4 blockade (Fig. 4). In a recent report, 4T1 tumor–bearing mice given α-galactosylceramide in a similar dose/schedule (100 ng every 4 days) in combination with anti-DR5 mAb to induce tumor cell apoptosis and anti-4-1BB mAb to provide costimulatory signals to T cells, showed significant rates of tumor rejection (45). This suggests that iNKT cell immunostimulatory function can be "rescued" by activation with α-galactosylceramide, but the outcome of this activation is influenced by the immunologic environment in which this takes place. Indeed, α-galactosylceramide has been shown to promote the regulatory functions of iNKT cells when given to mice with autoimmune diseases (11). Therefore, it will be important to determine which immunotherapy strategies may be successfully combined with α-galactosylceramide to elicit antitumor immune responses in cancer patients.

It has been proposed that there is a dichotomy between the two main subsets of NKT cells, noninvariant (or type II) NKT cells being sufficient for negative regulation of the antitumor immune response and iNKT cells being mostly responsible for protection (18). Our study shows that the selective absence of iNKT cells is sufficient to dramatically enhance the response to treatment with radiotherapy and CTLA-4 blockade in mice with an established metastatic mammary carcinoma. Importantly, at least in this tumor model, an antitumor immune response was detectable even in the absence of treatment, consistent with a role for iNKT cells in the suppression of antitumor immunity. Therefore, it is likely that in cancer, similarly to other autoimmune/inflammatory diseases (10), the regulatory role of invariant and noninvariant NKT cells is not absolute, but is influenced by both the immunologic environment of the tissue/tumor and unknown factors of the host. For instance, a glycolipid produced by melanoma cells has been shown to be cross-presented by dendritic cells (DC) and to induce production of IL-10 by NKT cells (46). The presence of iNKT cells in 4T1 tumors (Fig. 5) raises the question of whether they may recognize a tumor-derived glycolipid. Few endogenous CD1d ligands are currently known. Identification of CD1d ligands that are expressed by cancer cells and may be recognized by different NKT cell subsets and/or with different affinity will help clarify the role of NKT cells in regulation of antitumor immunity (47).

Previous studies have shown a marked enhancement of antitumor immunity in CD1d-deficient mice that lack both subsets of NKT cells (18, 21, 22), and these results have been interpreted as evidence of a strong regulatory role of type II NKT cells. However, the recent evidence that some mouse tumors express low levels of CD1d (48) raises the possibility that CD1d may act as a neoantigen in CD1d-deficient mice, similarly to *STAT6* in *STAT6*-deficient mice (49). Although CD1d expression in 4T1 cells has not been detected by immunostaining and flow cytometry (ref. 18 and data not shown), using a more sensitive technique, real time reverse transcription-PCR, we have found that 4T1 cells express CD1d (Supplementary Fig. S1). Because CD1d-reactive T cells may be present in the

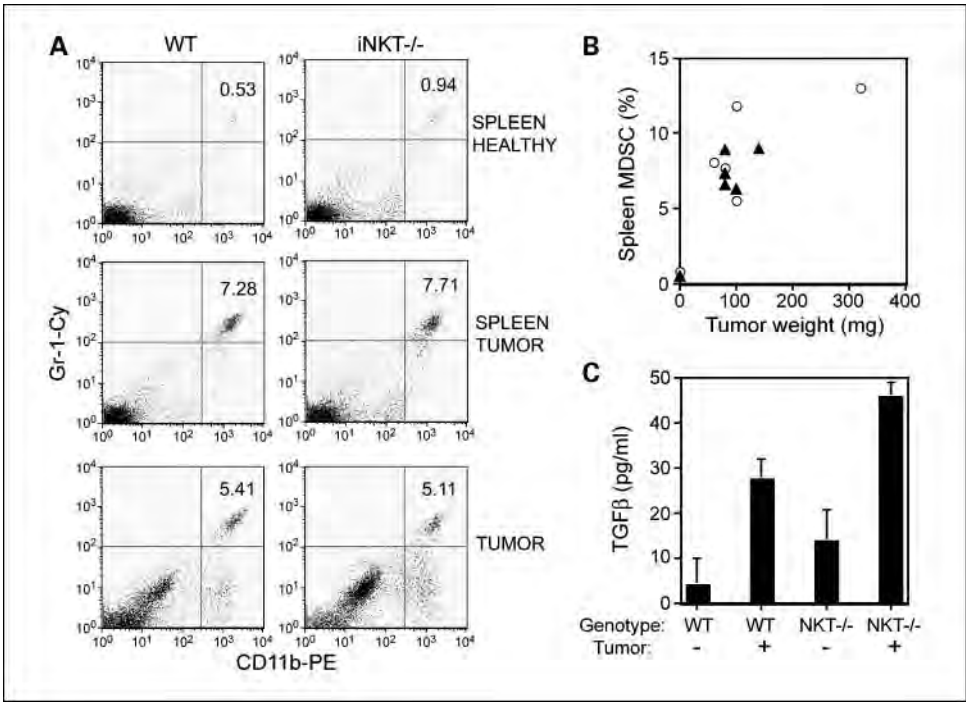

Fig. 6. Accumulation of MDSC and TGFβ production in wild-type and iNKT-/- mice bearing 4T1 tumors. Wild-type and NKT-/- mice were inoculated s.c. with 5×10^4 4T1 cells on day 0 (n = 5/group). Three non–tumor-bearing mice of each genotype served as controls. On day 14 primary tumors and spleens were harvested and obtained cell suspensions were stained with PE-anti-CD11b and Cy-anti-Gr-1 mAbs to detect MDSC. *A,* representative histograms of spleen from one healthy (*top row*) and one tumor-bearing (*middle row*) mouse showing the CD11b-positive Gr-1-positive MDSC. Histograms from pooled tumors isolated from 5 wild-type and 5 iNKT-/- mice showing the CD11b-positive Gr-1-positive MDSC (*bottom row*). Numbers indicate the percentage of MDSC. *B,* percentage of spleen MDSC in relation to the tumor weight in wild-type (*closed triangles*) and iNKT-/- (*open circles*) mice. Each symbol represents an individual mouse. For control healthy mice, one symbol representing the mean of 3 animals is shown. *C,* splenocytes isolated from tumor-free (-; n= 3) and tumor-bearing (+; n = 5) mice were cultured o.n. and the concentration of TGFβ in the culture supernatants was determined by ELISA. Data are the mean ± SE. In both wild-type and iNKT-/- mice spleen cells derived from tumor-bearing mice produced significantly more TGFβ than spleen cells derived from healthy mice ($P < 0.05$). Although the baseline production of TGFβ did not differ significantly between wild-type and iNKT-/- healthy mice (P = 0.35), tumor-bearing iNKT-/- mice produced more TGFβ than tumor-bearing wild-type mice ($P < 0.01$).

repertoire of CD1d-deficient mice, their possible role in 4T1 tumor rejection will need to be addressed.

In conclusion, we show that mice developing a detectable antitumor response to a syngeneic poorly immunogenic tumor because their regulatory circuits are "slightly" altered in the absence of the iNKT cells, only show a mild improvement in survival in the absence of treatment. However, their response to immunotherapy is dramatically improved in comparison with wild-type mice. These results model clinical observations that patients with preexisting antitumor immune responses are more likely to respond to immunotherapy (50). Although the intrinsic tumor immunogenicity has often been invoked to explain these responses, focusing on the "immunologic" make-up of the host may be important for an improved understanding of the determinants of response to immunotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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APPENDIX 2

Pilones, KA, et al ***Immunoregulation by Invariant Natural Killer T Cells in a Mouse Model of Metastatic Breast Cance***r
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Immunoregulation by Invariant Natural Killer T Cells in a Mouse Model of Metastatic Breast Cancer

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Immunoregulatory and suppressive mechanisms are major obstacles to the success of immunotherapy in cancer patients. Invariant natural killer T (iNKT) cells have been shown to down-regulate the immune response in autoimmune diseases, but to up-regulate anti-tumor immunity when activated by α-galactosylceramide (α-GC). We have previously shown that the combination of radiotherapy (RT) to the primary tumor and CTLA-4 blockade induces a CD8-mediated anti-tumor response inhibiting metastases and extending the survival of mice bearing the poorly immunogenic and highly metastatic 4T1 mammary carcinoma. Surprisingly, the response to treatment was markedly enhanced in the absence of iNKT cells, with 50% of iNKT cells-deficient (iNKT $-/-$) mice versus 0% of WT mice showing complete tumor regression, long-term survival, and resistance to a challenge with 4T1 cells. Activation of iNKT cells by α-GC administration to wild type (WT) mice with established 4T1 tumors did not enhance the response to treatment. In the absence of treatment, tumors grew similarly in WT and iNKT $-/-$ mice. Repeated vaccination with irradiated tumor cells did not induce protective immunity in either WT or iNKT $-/-$ mice, indicating that the tumor is not differentially immunogenic in iNKT $-/-$ mice. However, spontaneous lung metastases were reduced in iNKT $-/-$ as compared to WT mice, and this difference was eliminated by depletion of CD8 T cells, suggesting that development of anti-tumor CD8 T cell responses is enhanced in the absence of iNKT cells. Interestingly, whereas WT and iNKT $-/-$ mice showed a similar systemic and intratumoral increase in myeloid-derived suppressor cells (MDSC), the numbers of intratumoral CD11c+ dendritic cells (DCs) was significantly higher in iNKT $-/-$ mice ($P < 0.001$) Furthermore, even in the absence of treatment, DCs obtained from tumor and tumor-draining lymph nodes of iNKT $-/-$ mice showed increased expression of maturation markers (CD40, CD80, CD86, MHC-II) compared to DC from WT mice. This data suggests that iNKT cells may negatively regulate cross-priming of anti-tumor CD8 T cells at the level of the antigen-presenting cells in this tumor model. Supported by DOD BCRP Postdoctoral Award BC086964 to KP.

Generation of an Innate Immune Microenvironment as a Novel Mechanism for Myotoxins to Potentiate Genetic Cancer Vaccines

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We recently reported that administration of low doses of myotoxins at vaccination sites potentiated antigen-specific T-cell immunity and tumor protection induced by genetic lymphoma idiotype vaccines in mice, an effect which was superior to TLR agonists. In the current study, we found unexpectedly that the mechanism of this potent adjuvant effect was immune-mediated. Myotoxins induced sterile inflammation at vaccination sites, associated with a predominant infiltration of dendritic cells (DC). Inhibition of DC recruitment abrogated the immune stimulation effect of myotoxins, suggesting the requirement for DC. Genetic profiling of myotoxin-treated tissues revealed characteristics of an immune microenvironment with upregulation of chemokines, proinflammatory cytokines, Toll-like receptors (TLR) and their endogenous ligands, and activation of innate immunity. Mechanistic experiments in vivo also elucidated the requirement for genes triggering DC maturation including TLR signaling and CD40. These studies suggest that myotoxins-induced sterile inflammation generates a favorable innate immune microenvironment that promotes multiple stages in the development of adaptive immunity. This novel mechanism of immune potentiation may be exploited for development of adjuvants for genetic vaccines against cancer.

Interferon-β Secretion in the Tumor Microenvironment can Cause Potent Tumor Control Through Host Cells Independently from Adaptive Immunity

Robbert M. Spaapen, Mercedes B. Fuertes, Justin P. Kline, Yan Zheng, Thomas F. Gajewski. *University of Chicago, Chicago, IL.*

Innate immune recognition of tumors is essential for generating a natural adaptive anti-tumor immune response. We have recently shown that host interferon-β (IFN-β) production is required to generate a primary adaptive immune response against B16-F10 melanoma and other murine transplantable tumors. This fundamental observation generated the hypothesis that provision of exogenous IFN-β in the tumor microenvironment might augment spontaneous adaptive immune responses even further, perhaps to the point of promoting complete tumor rejection. To test this notion, we retrovirally transduced the murine IFN-β cDNA into B16-F10 melanoma cells, which expressed the model SIYRYYGL (SIY) antigen to enable monitoring of T cell dynamics. In an autocrine manner, these cells subsequently secreted the chemokine CXCL10 and upregulated MHC class I surface expression, supporting a positive immunomodulatory effect. Upon implantation into C57BL/6 mice in vivo, IFN-β expressing B16-F10 tumors initially grew but then were potently controlled, even at doses up to 6×10^6 cells. A mixed population of wildtype and IFN-β-expressing B16-F10 melanoma cells was also completely controlled. Moreover, 10-day established B16-F10 tumors were completely rejected after intratumoral injection of IFN-β-expressing B16-F10 melanoma cells, arguing for a potent bystander effect. Expression of the type I interferon receptor (IFNAR1) on host cells was required to mediate this suppression of tumor growth. However, tumor control was not associated with significantly increased T cell responses as measured by IFN-γ ELISPOT or tetramer analysis specific for the SIY antigen, suggesting that improved adaptive immunity might not be the mechanism at work. Strikingly, identical tumor control was observed in Rag2$^{-/-}$ mice, arguing that T cells and B cells were dispensable. In addition, mice depleted of NK cells also demonstrated control of IFN-β-expressing tumors. Interestingly, IFN-β-expressing tumors showed a massive increase in macrophage infiltration in the tumor microenvironment, which may be immune effectors in this setting. Therefore, local IFN-β expression in the tumor microenvironment can mediate strong anti-tumor effects mediated by the host, independently of T, B, or NK cells.

Adenovirus-Engineered Human Dendritic Cells Effectively Recruit Natural Killer Cells via CXCL8/IL-8 and CXCL10/IP-10

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Recombinant adenovirus-engineered dendritic cells (Ad.DC) are an effective modality for inducing anti-viral and anti-cancer T cell immunity. The effectiveness of Ad.DC-based vaccines may depend on the ability of Ad.DC to crosstalk with natural killer (NK) cells and to activate, polarize, and bridge innate and adaptive immunity. Previously we reported that human Ad.DC can efficiently activate NK cells by cell-to-cell contact. In order for this interaction to occur, Ad.DC must be able to effectively attract NK cells. In the present study, we evaluated the ability of Ad.DC to recruit resting NK cells in vitro. We found that Ad.DC effectively recruited both CD56hi CD16- and CD56lo CD16+ NK cell subsets. Resting NK cells consistently expressed CCR7 (CCL19/MIP-3β receptor; both subsets), CXCR1 (CXCL8/IL-8 receptor; CD56lo CD16+ subset) and CXCR3 (CXCL9/MIG and CXCL10/IP-10; both subsets), while Ad.DC secreted high, moderate and minute levels of CXCL8/IL-8, CXCL10/IP-10, and CCL19/MIP-3β, respectively, but no CXCL9/MIG. These findings suggested that CXCL8/IL-8 and CXCL10/IP-10 were the most likely ligands involved in chemotaxis.